Aging and Disease
www.aginganddisease.org
Volume 2, Number 5; 361-373, October 2011

Review

Aging Impairs Murine B Cell Differentiation and Function in Primary and Secondary Lymphoid Tissues

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[Received June 15, 2011; Revised August 4, 2011; Accepted August 5, 2011]

ABSTRACT: Age-related changes in humoral immunity are responsible for the reduced vaccine responses observed in elderly individuals. Although aging has been shown to affect T cells, dendritic cells and macrophages and these effects significantly impact humoral responses, intrinsic alterations in B cells also occur. We here provide an overview of age-related changes in mouse B cells. In particular, we summarize data from the literature showing age-related changes in B cell differentiation in the bone marrow, in B cell marker expression and cell survival in the periphery and in the ability to make specific antibodies in both splenic and mucosal tissues. Moreover, we summarize the results from our studies showing that the ability to undergo class switch recombination, the enzyme activation-induced cytidine deaminase and the transcription factor E47 are all decreased in stimulated B cells from old mice. The defects presented in this review for aged B cells should allow the discovery of strategies for improvement of humoral immune responses in both humans and mice in the near future.

Key words: Aging; Differentiation; Lymphoid tissue; Function; Immunity

There is an inverse relationship between immune status, response to vaccination, health and longevity, suggesting that the immune system becomes less effective with advancing age. Both humoral and cellular immune responses have been shown to be decreased in aged humans and experimental animals [1-6], leading to increased frequency and severity of infectious diseases and reduced protective effects of vaccination. Hospitalization due to infection is much more common in the elderly than in younger individuals and has been identified as a major contributor to the development of disability in elderly individuals [7]. This decline in physical activities and consequent disability represent a significant economic burden due to both direct (medical) and indirect costs (inability to work, reduction in productivity).

The aging immune system

Despite intensive research work, many of the basic mechanisms of age-related immune dysfunction have yet to be clarified. The observation that antibody responses to T-dependent (TD) antigens are significantly reduced in old mice, whereas those to T-independent (TI) antigens are maintained, has suggested that the age-related decline in antibody responses can be largely attributed to the senescence of T cell functions. The total number of anti-dinitrophenyl (DNP) plaque-forming cells generated in the spleen of old mice immunized with the TD antigen DNP-bovine gamma globulin (BGG), have been reported to be significantly reduced in old as compared to young mice [8], whereas old mice immunized with the TI antigens DNP-Ficoll [9] or type III pneumococcal

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ISSN: 2152-5250

polysaccharide (SIII) and LPS [10] were able to make as many anti-DNP plaque-forming cells as young mice did. The contribution of decreased T cell function with age to the reduction in somatic hypermutation and to shifts in the repertoire has also been reported [11-13]. CD4 T cells from old mice have been shown to produce less IL-2, to proliferate poorly and differentiate less well upon antigen stimulation [14]. As a consequence of reduced IL-2 production, CD4 T cells from old mice may have reduced CD40L expression, which is a crucial molecule for cognate B/T interaction [15]. It has recently been shown that pro-inflammatory cytokines can enhance helper activity of CD4 T cells from aged mice, and up-regulate humoral responses, when delivered together with the antigen *in vivo*. This occurs because they induce a heterogeneous population of CD4 T cell effectors which secrete Th1, Th2 and Th17 cytokines. In particular, the adjuvant activity of pro-inflammatory cytokines is likely a result of their ability to enhance proliferation and IL-2 production by helper T cells as well as their ability to enhance the expression of CD40L [16]. Although these results provide a proof of principle that the addition of pro-inflammatory cytokines to a vaccine preparation can significantly enhance the cognate helper activity of CD4 T cells from young and especially old mice, by broadening the profile of cytokine secretion by helper T cells, this approach cannot be recommended in elderly humans already showing high levels of inflammation which in many cases is a predisposition for autoimmunity.

A defect in the generation of CD4 memory cells from old naive cells has also been reported. This defect could account for reduced antigen-specific B cell expansion, germinal center (GC) development, generation of memory B cells and IgG production observed in aging [17]. CD4 T cells are also important for the development of functional CD8 memory T cell populations [18, 19], which regulate immunity against intracellular pathogens, such as viruses, leading to the rapid generation of highly functional effectors that can kill infected cells upon a second encounter with the specific pathogen [20]. One of the most dramatic changes in the memory T cell population during aging is the appearance of CD8+ clonal expansion and loss in CD28 expression [21, 22], which is associated with an increase in cytomegalovirus positivity [22, 23]. Unsuccessful vaccination has been positively correlated with the expansion of these T cells [24].

The number of CD1d-restricted TCR+ NKT cells increases with age [25]. These cells also contribute to the age-associated decline of T cell immunity, and indirectly to B cell immunity because they significantly suppress the expansion of antigen-specific CD4 T cells. T regulatory cells (Tregs) also act similarly. Tregs act in a cell contact-dependent but cytokine-independent way, by inducing cell death which is mediated by a granzyme-dependent, partially perforin-dependent pathway [26]. In addition direct suppression of B cells by Tregs without the need to first suppress Th cells has been shown[27]. T cell-mediated suppression is increased with age [28]. In particular, there are significantly more CD4+CD25+FoxP3+ Tregs in the spleen and lymph nodes of old mice as compared with the young ones. Further studies are needed to completely understand the reason why Tregs accumulate or expand during aging.

Dendritic cells (DCs) from old mice, unlike DCs from elderly individuals [29], are less able to stimulate T and B cells [30]. Follicular DCs (FDCs), critical to the formation of plasma cells in the GCs of secondary lymphoid organs and generation of high affinity antibodies, have also been found less effective in old as compared to young mice. This seems to be due to the reduced expression of both CD21, and FcγR which are involved in the trapping of immune complexes and in signaling [31]. Moreover, reduced FDC function could result in reduced persistence of antigen on the dendrites and, in turn, reduced maintenance of functional memory B cells. This may help explain why specific antibody responses are dysregulated in old mice and humans.

Macrophages from old mice have impaired respiratory burst and reactive nitrogen intermediates as a result of altered intracellular signaling, which renders them less able to destroy bacteria [30]. Reduced antigen presentation function has been reported and attributed to decreased expression of MHC class II molecules [32-34]. Moreover, the amounts of TLRs have been shown to decrease [35, 36] or not [37] with age, whereas the secretion of various chemokines and cytokines are decreased [35, 37]. The production of prostaglandin E_2 (PGE_2) and PGJ2 by macrophages are increased in old mice [31, 38]. PGE_2 alters DC function by blocking the production of IL-12, therefore causing less T stimulation. PGJ2 is a potent stimulus for CXCL2, a chemokine that regulates infiltration of inflammatory leukocytes in inflamed tissues.

Although several studies have provided strong evidence that both T cells and antigen-presenting cells (APCs) significantly contribute to the age-related decline of antibody responses, intrinsic alterations in B cells have also been shown with age in mice [39-42] and humans [43-45]. Except where specifically indicated, this review deals with studies in mice.

Age effects on B cells

The generation of B2-B cells or "conventional B cells" from bone marrow precursors is impaired in old mice. In particular, there is a decreased production of pro-B and pre-B cells in the bone marrow [46-48]. Earlier B cell precursors are also diminished in old mice, including both stem cells [49, 50] and common lymphoid progenitors (CLPs) [47, 51]. Conversely, B1-B cell progenitors are retained in the bone marrow of old mice even when B2-B cell precursors are reduced [46]. However, the murine peripheral mature B cell pool does not change in size with age, but changes in the relative proportions of the different cell subsets occur (see below).

In contrast with mouse B cells, the absolute numbers of human B cell precursors in the bone marrow have been shown to decline [52] or not [53] with age, whereas peripheral B cell percentages and numbers significantly decrease with age [44, 54, 55]. As to B cell subsets, it has been shown that naïve B cell numbers but not percentages, and total switch memory (both CD27+ and CD27-) B cell percentages and numbers are significantly decreased by age [44, 55-57]. The percentages of IgM memory B cells are unchanged by age but the absolute numbers are significantly decreased [44, 55-57]. In the literature others have found an increase in the percentage but a decrease in the number of total memory B cells [58, 59]. This increase in the percentage of total memory B cells can be due to an increase in the percentage of IgM memory B cells, which represents the major memory B cell subset. Our results below clearly indicate that not only the numbers of switch memory B cells decrease with age but also the function of class switching B cells. This finding is significant especially for specific responses, i.e. anti-influenza vaccine response.

Despite reduced output of B cell precursors, the population of mature splenic B cells is maintained in old mice mainly because of their increased life-span [50]. Studies in mice have indeed shown that the numbers of follicular (FO) B cells remain constant with age, suggesting that they turn over more slowly in old than in young mice, as indicated by *in vivo* labeling studies [60-62].

Reduced B cell generation from the bone marrow has been suggested to affect the "read-out" of different B cell repertoires with age, homeostasis of particular peripheral B cell subsets [63] and in turn humoral immune functions. The peripheral B cell pool is enriched with cells that are long-lived at least in part as a consequence of chronic stimulation by environmental antigens [64]. Increased numbers of B cells with autoreactive specificities and increased amounts of serum autoantibodies have been reported in old mice [65]. The antigen experienced B cell pool include B1-B cells, marginal zone (MZ) B cells, memory B cells and B cells with characteristics of chronic activation. B1-B cells might either accumulate or expand with age as a result of chronic stimulation by environmental antigens [64]. In C57BL/6 mice, the MZ pool also enlarges with age [64], whereas in BALB/c mice it decreases [39, 66]. Because MZ B cells display repertoire skewing similar to B1-B cells, the expansion of these cells, at least in some cases, might help to explain the appearance of polyreactive and autoreactive antibodies. Recently, another mature B cell subset that accumulates with age has been described by two groups and called age-associated B cells (ABC) as they represent up to 30% of the peripheral B cell pool in C57BL/6, BALB/c, (BALB/c x C57BL/6) F1 and DBA/2 mice 22 months of age or older [67, 68]. The first group [67] has shown that these double negative (CD19+AA4.1-CD43-CD21-CD23-) B cells are refractory to BCR and CD40 stimulation, but they respond to TLR9 or TLR7 stimulation and divide when stimulated upon combined BCR and TLR ligation, leading to Ig production and preferential secretion of IL-10 and IL-4. Moreover, ABC can be derived from FO B cells following exhaustive expansion *in vitro* and *in vivo*. The second group [68] has shown that this population (CD19+CD11b+CD11c+CD21-IgD-) is increased not only in old female C57BL/6 but also in young lupus-prone NZB/WF1 mice and the equivalent cells can also be detected in the peripheral blood of elderly women with autoimmune diseases. Upon stimulation, ABC both from autoimmune-prone and healthy old mice secrete autoantibodies and depletion of these cells *in vivo* leads to reduction of autoreactive antibodies, suggesting that the cells might have a direct role in the development of autoimmunity. Although these results on age differences in B cell subsets suggest a shift in functional primary B cell subsets does occur and may help to account for at least some of the overall features of humoral immunosenescence, more characterization and functional studies are necessary.

The peripheral B cell pool is also regulated by competition for the survival factor BAFF/BLyS [50]. BAFF and its receptors mediate peripheral B cell homeostasis. The size, dynamics and behavior of the B cell subsets influenced by BAFF change with age [50] and enhanced BAFF responsiveness may contribute mechanistically to the increased lifespan and decreased turnover rates of the aged B cell pool. FO and MZ B cells rely on BAFF/BLyS for survival, but ABC do not,

although they express BAFF/BLyS receptors and sequester this cytokine [67].

Ability to make an optimal antibody response to exogenous antigens and vaccines declines with age in humans and animal models [42-44]. The changes in the humoral immune response with age are both qualitative and quantitative: reduced serum concentrations of antigen-specific Ig, antibody specificity, affinity, and class switch recombination (CSR) being changed. In particular, a progressive decline in both the number and the size of GCs has been reported [13, 15]. The impairment in GC reactions occurring during aging results not only from T cell and FDC defects but also from intrinsic B cell defects, for example postulated decreased somatic hypermutation (SHM) of Ig genes. This results in decreased antibody affinity maturation, switch memory B cells and plasmablasts upon immunization in the elderly. There is also diminished recirculating antibody-secreting plasma cells in the bone marrow [69]. In adoptive transfer experiments, plasma cells producing both low and high affinity antibodies as a consequence of a recent antigenic stimulation were found to be significantly diminished in the bone marrow of old as compared to young mice [70].

The effects of age on antibody affinity maturation are controversial and results published by different groups are conflicting. Briefly, SHM in the IgH V region genes of GC B cells was found to be reduced in splenic GCs [71] or increased [72] in Peyer's patch GCs of aged mice and the reduction was attributed to defective T cell help to B cells *in vivo* [15]. However, when adoptive hosts receiving young T cells and aged B cells were tested, they also exhibited a reduced capacity to undergo SHM of antibody genes, suggesting a deficiency in the B cell compartment as well [73].

CSR, the DNA recombination process required for the generation of switched antibodies, is decreased by age. This decrease, observed in both mice [42] and humans [43, 44], produces less IgG for an optimal newly generated antigen response; not only are the IgG constant region effector functions critical for an optimal response but the switch and maturation to IgG is also associated with SHM and affinity maturation of the V region, both dependent on AID. CSR requires chromatin opening of a particular switch (S) region, and is mediated by cytokine-induced germline transcription. In this process, AID is required [74, 75]. AID initiates CSR by deamination of cytidine residues in switch (S) regions, thus creating uracils, and the resulting mismatches are recognized by specific enzymes and excised, leading to DNA double strand breaks [76, 77]. E2A activity is necessary for CSR because the E47 transcription factor has been shown to be important in transcriptional regulation of *Aicda*, the gene encoding AID [78]. E47 is a class I basic helix loop helix protein, able to bind with relatively high affinity to the palindromic DNA sequence CANNTG, referred to as an E-box site [79-81]. E-boxes have been found in the promoter and enhancer regions of many B lineage-specific genes and regulate a large number of processes involved in B cell commitment and differentiation [82-86].

The decreased ability of old B cells to undergo CSR *in vivo* and *in vitro* likely results from combined age-related defects in T cells, B cells, and antigen-presenting cells. It has been argued that age-defects in T cells and APCs are sufficient to explain the reduced antigen-specific B cell expansion, GC development and IgG production observed during aging [17]. However, work from our laboratory has unequivocally demonstrated intrinsic B cell defects in aged mice [39-42] and humans [44]. Briefly, *in vitro* stimulated splenic B cells from old BALB/c mice are deficient in CSR and secondary Ig production, largely due to decreased induction of E47 and AID [42]. Both E47 protein and mRNA levels are decreased after stimulation of splenic B cells from old mice as compared with young mice. As a consequence of E47 reduction, AID mRNA was also reduced to the same extent in old stimulated B cells [42]. We found that E47 and AID were decreased in old *versus* young B cells in response to various stimuli representing TD and TI independent pathways, such as anti-CD40/IL-4 [42], BAFF/IL-4 [87], or LPS [40]. As a consequence of the decrease in AID levels in old *versus* young B cells, less class switch DNA products were obtained 5 days after stimulation, whereas germline transcripts were indistinguishable in young and old B cells. These data are consistent with the defect in old B cells occurring at the CSR event and not due to problems with accessibility, or with cytokine signaling pathways leading to DNA accessibility. Decreases in E47 with age, in addition to directly decreasing levels of AID, may also directly affect SHM of the Ig loci via decreased transcription [88] or via other mechanisms [89].

In order to better demonstrate the direct connection between E47, AID, and CSR, we performed experiments with young and old $E2A^{+/-}$ (heterozygous, HET) mice. Young HET mice had half the level of CSR as compared to wild type controls. Old HET mice had reduced their CSR by another 2-fold, showing an additive effect of E2A reduction genetically and due to age. These experiments used C57BL/6 mice where the

age difference is less than that seen with B cells from young and old BALB/c mice (about 4-5 fold) and at this time it is unclear to what the strain difference is due [42].

NF-κB is another transcription factor which positively regulates CSR [90]. In our experiments, NF-κB (p65) nuclear protein levels were found comparable in young wild-type and HET mice as well as in old wild-type and HET mice, but old B cells had less (about half) p65 than young B cells [40] after LPS stimulation. In response to LPS, which does induce NF-κB, the difference in AID appears to track with the levels of E47, not NF-κB. If NF-κB were a major regulator of AID, here in both young and old, then the AID values for wild type and HET mice should have been more similar [40]. These results altogether demonstrate that aging down regulates E47 mRNA and protein levels, which in turn causes less AID, leading to less Ig class switch which affects the quality of antibody produced.

It has recently been demonstrated that a putative regulatory region in the *Aicda* gene contains both E2A- and Pax-5-binding sites, and the latter site is indispensable for AID gene expression [91]. Pax-5 is a transcription factor critical for B cell lineage commitment, B cell development and CSR in GC B cells [91, 92]. Moreover, Pax-5-dependent repression of X box binding protein-1 (XBP-1) is critical for inhibiting plasmacyte differentiation in the GC [93]. Interestingly, Pax-5 was found to be recruited to the AID locus when AID transcription was initiated. E2A proteins have been described to regulate Pax-5 not directly but through their regulation of EBF [94]. Consistent with these observations is the finding that the Pax-5 promoter contains functional EBF binding sites [95]. Like E47, Pax-5 DNA binding activity has been shown to be strongly reduced in splenic B cells from old mice [96]. However, decreased Pax-5 binding activity was not the result of decreased levels of Pax-5 mRNA, as shown by RNase protection assays [96, 97], suggesting that either post-transcriptional or post-translational mechanisms may be responsible for the down-regulation of Pax-5 proteins in aged B cells. Our laboratory is currently investigating these mechanisms.

Molecular mechanisms for reduced class switch in B cells from old mice

A mechanism we have found for the age-related decrease in E47 levels in old splenic B cells is decreased mRNA stability [41]. E47 protein degradation rates are comparable in young *versus* aged splenic B cells, in contrast with our results in bone marrow-derived IL-7-expanded pro-B/early pre-B cells [98]. Thus, different molecular mechanisms to accomplish decreased E47 are seen during aging in different differentiation stages of the B lineage.

The stability of labile mRN, e.g.E47 in splenic B cells from old mice, may be controlled by signal transduction cascades, where the final product of the cascade phosphorylates a protein that interacts with adenylate/uridylate-rich elements (ARE) in the 3' untranslated region (UTR) of mRNA and modifies its stability [99]. ARE sequences have been found in the 3'-UTR of many mRNAs, including E47. The E47 mRNA contains the pentamer sequence AUUUA in the genomic poly-A end of the 3'-UTR plus several U-rich and AU-rich elements.

The stability of E47 mRNA is regulated at least in part by the p38 MAPK signal transduction cascade, which phosphorylates a protein, tristetraprolin (TTP), that interacts with the ARE sequences in the 3'-UTR of many mRNAs [100]. The phosphorylation of TTP prevents its binding to ARE sequences.TTP binds the 3'-UTR of its own mRNA, which also contains AREs and stimulates deadenylation which leads to mRNA degradation [101, 102]. The TTP protein is a low-abundance cytosolic protein whose levels are dramatically induced by LPS, but phosphorylation via p38 MAPK is also increased so the net effect is not more TTP activity. This is true in young B cells but not in old (see below). The protein is stable once induced, in contrast with its labile mRNA [103-105]. TTP in purified splenic murine B cells is induced not only by LPS stimulation [39], but also by other stimuli, such as LPS/IL-4, anti-CD40, anti-CD40/IL-4, or IL-4. In all cases, the levels of TTP mRNA and protein in old B cells were significantly higher as compared with those in young B cells. LPS was the best stimulus, but LPS/IL-4 and anti-CD40/IL-4 are also good stimuli to induce TTP. The different levels of expression of TTP in young and old B cells stimulated with LPS or with anti-CD40/IL-4 is consistent with different E47 mRNA expression in young and old B cells observed before [42].

We have found that TTP is involved in the degradation of the E47 mRNA [39]. To demonstrate this, we performed a series of experiments in which the mRNA from stimulated young B cells was incubated with cytoplasmic extracts of activated B cells from young and old mice. Subsequently, E47 mRNA levels were revealed by qPCR. Results clearly indicate that cytoplasmic lysates from old B cells induced more degradation of E47 mRNA than those from young B cells. When TTP was removed from both the young and old cytoplasmic lysates by immunoprecipitation

with specific anti-TTP antibody before the interaction with the mRNA occurs, the expression of E47 mRNA was significantly increased in both cases, but much more increased when TTP was removed from the old lysates [39]. In these experiments, poly(A) minus RNAs (including small RNAs) were removed, and only mRNA was present in the mixture with proteins. Inhibition of the p38 MAPK signaling pathway significantly reduces TTP protein phosphorylation in B cells. Old B cells in response to LPS make less phosphorylated p38 MAPK [39] and therefore, as would be expected, make less phosphorylated TTP. This leads to an increase in the amount of TTP bound to the 3'-UTRs of E47 (and inflammatory cytokines, such as TNF-α) and therefore decreased E47 mRNA stability in old B cells.

More recent results from our laboratory [40] have demonstrated that the levels of PP2A, a serine/threonine protein phosphatase that plays an important role in the regulation of several major signaling pathways, is increased in old B cells. Also PP2A phosphatase activity is increased in old B cells. As a consequence of this higher phosphatase activity in old B cells, p38 MAPK is less phosphorylated as compared with young B cells. PP2A dephosphorylation of either p38 MAPK and/or TTP may account for more binding of the hypophosphorylated TTP to the 3'-UTR of the E47 mRNA, inducing its degradation. Our data support this mechanism for regulating the molecular pathways that lead to reduced antibody responses in aging.

We further confirm and extend our results on the involvement of 3'-UTR sequences on the E47 mRNA degradation by showing that the rescue of CSR in old B cells can be accomplished by transducing primary B cells with a retroviral construct containing E47 without the 3'-UTR [106]. Briefly, splenic purified B cells from old mice overexpressing a stable E47 mRNA significantly up-regulated E47, AID, and CSR and improved B cell responses, suggesting that at least in principle it is possible to rescue the intrinsic B cell defects and the humoral response observed in old B cells. Moreover, in another series of experiments, a retroviral construct containing DsRED with either the E47 or ku80 3'-UTR at its 3' terminus was designed. The ku80 mRNA is very stable and is not differentially regulated in young and old splenic B cells [41]. After transducing the BJAB cell line (human Burkitt B-cell lymphoma) with the DsRED control vector, DsRED-E47 3'-UTR or DsRED-ku80 3'-UTR, it was observed that the DsRED-E47 3'-UTR had significantly less DsRED mRNA than the DsRED vector. These results altogether clearly demonstrate that the E47 3'-UTR confers instability of a target mRNA in a B cell line and also in primary B cells from old mice, but not in a non-B cell line (MCF-7).

B cell function in the TNF-α-enriched environment of old mice

The inflammatory status of the animal may impact B cell function. B cells are affected by the cytokines and other factors released by cells of both innate and adaptive immune systems. Moreover, B cells themselves express innate immune receptors which recognize exogenous pathogens or the adjuvants used to induce an immune response. Therefore, B cells can either promote immune responses by acting as cellular adjuvants or they can regulate immune responses by secreting immunoregulatory cytokines.

Aging is characterized by a dysregulation of inflammatory and anti-inflammatory networks, which results in a low grade chronic pro-inflammatory status called inflammaging [107]. The age-related increase in circulating inflammatory mediators such as cytokines and acute phase proteins are markers of the low-grade inflammation observed with aging. Age-related alterations in responses to immune stimulation, for example chronic T cell stimulation with viruses such as CMV, also contribute to low-grade inflammation by increasing the level of pro-inflammatory mediators such as TNF-α [108]. Although production of pro-inflammatory cytokines is thought to be in part a macrophage-mediated event, other cell types, including stroma (i.e. epithelium, endothelium, fat) and T cells, produce these mediators *in vivo*. B cells, through the secretion of cytokines such as TNF-α, have been shown to contribute to immunity against infectious agents, such as *Toxoplasma gondii*, *Heligomosomoides polygyrus* or *Pneumocystis carinii* by promoting expansion and differentiation of primary and memory Th1 [109] and Th2 cells [110, 111]. This pro-inflammatory role of B cells may support their pathogenic role in a wide range of autoimmune diseases [110]. B cell depletion has indeed been shown to be effective in the treatment of several T cell-mediated autoimmune diseases, such as Multiple Sclerosis, Type-1 Diabetes and Rheumatoid Arthritis. However, the efficacy of the therapy does not correlate with changes in the levels of circulating autoantibodies, strongly supporting the major role of B cells in autoimmunity as regulatory rather than antibody producing cells [110].

TNF-α can positively or negatively modulate immune responses. In general, inflammation is a protective response of the body to infection. However,

increased plasma levels of TNF-α, which contribute to the chronic, low-grade inflammation typical of old age, can have deleterious effects as are implicated in the pathogenesis of several disabling diseases of the elderly [112-116].

Similar to elderly humans, old mice have plasma levels of TNF-α higher than young mice [117] [Frasca et al, submitted]. The production of TNF-α by mouse and human B cells and its role on B cell function has not yet been investigated. Recent data from our laboratory [Frasca et al, submitted] have shown that the pre-incubation of splenic purified B cells with exogenous TNF-α before LPS stimulation significantly decreases both young and old B cell responses. Moreover, blocking TNF-α by adding an anti-TNF-α antibody to LPS-stimulated B cells significantly restores CSR in young and more significantly in old cultured B cells. The molecular mechanism for TNF-α-mediated down-regulation of CSR in old B cells was shown to be associated with increased TTP. These results reveal a new molecular mechanism which may contribute to reduced antibody responses in aging and potentially other conditions of increased chronic inflammation. Studies are needed to better characterize the effects of newly identified molecular biomarkers of aging, such as TNF-α, which may be responsible for the reduced response of B cells from elderly individuals to vaccines.

Aging impairs mucosal immunity

The gastrointestinal tract of old animals and humans is particularly susceptible to infectious diseases because dramatic changes in mucosal immunity occur with age. Age-associated changes in the intestinal microbiota have also been reported [118-120], in particular a reduction in the numbers and species of protective anaerobes, suggesting that the quality of IgA responses may be altered. This is important considering that pathogen-specific sIgA in the mucosa is the first line of defense in reducing invasion, dissemination and/or growth of bacteria and viruses [121]. For this reason, the effect of aging on the composition of the gut microbiota and the effects that these age-related changes can have on the health status have recently received considerable interest, with the goal of improving the wellness of elderly individuals by modulating the balance of the gut microbiota with food.

Earlier studies conducted in BALB/c mice reported a decline in the number of both T and B cells in the mesenteric lymph nodes, in the Peyer's patches (PP), but not in the lamina propria (LP), and a decline in the *in vitro* proliferative capacity and CSR in response to TD antigens, but not to TI antigens. However, IgA production in the intestinal lumen was found only slightly decreased by aging [122]. The constancy of intra-luminal IgA levels could be of physiological significance in host defense at the gut mucosal surface in old mice. Another old study conducted in C57BL/6 mice has shown impaired mucosal B and T cell functions during an antigen-specific response, arising as early as 12 months of age, at the protein, cellular, and molecular levels [123]. More recent studies have shown that old rats [124] or rhesus monkeys [125], orally immunized with native cholera toxin from *Vibrio cholerae* (nCT), produce significantly lower levels of specific IgA than their younger controls. Old mice orally immunized with *Haemophilus influenzae*, conjugated with diphtheria toxoid and nCT as mucosal adjuvant, produce less IgA specific antibodies that young controls [126].

Synthetic oligodeoxynucleotides containing immunestimulatory CpG motifs (CpG-ODN) were suggested to be effective Th1 adjuvants when delivered by mucosal or systemic immunization routes in young mice [127]. In fact, when young and old mice were orally immunized with OVA plus CpG ODN as adjuvant, a comparable OVA-specific systemic IgG and mucosal IgA production were observed [128-130]. Moreover, when young and old mice were nasally immunized with OVA plus a nasal adjuvant containing a plasmid encoding the Flt3 ligand coupled to CpG ODN, comparable levels of specific IgA responses were induced in nasal secretions of young and old mice [131], suggesting that the mucosal delivery of CpG ODN as adjuvant offers an attractive possibility for the development of an effective mucosal vaccine for older individuals.

These results altogether clearly indicate that aging decreases mucosal antibody responses. It is not clear however if the effect of aging is on the generation of plasma cells from PP plasmablasts or on the migration of these plasmablasts to the intestinal LP. Local IgA production in the intestinal LP appears to be under the regulation of IL-6, whose levels increase with age [132]. It has been proposed that the reduction in IgA plasma cells in the intestinal LP of old mice might reflect impaired B cell differentiation due to reduced sensitivity of these cells to IL-6 [133]. In addition, impaired antigen presentation by mucosal DCs [121], cytokine/chemokine production by mucosal CD4+ T cells [134] and increased numbers of T cells with regulatory function [135] have been reported and may account for the suppression of either terminal

differentiation of B cells or antibody production in the intestinal LP.

Can B cell function be improved in old age and how?

Because intrinsic defects in cells of the immune system have been extensively reported, efforts to increase the efficacy of vaccination in old age have been made. Most of the studies published have reported results on the restoration of T cell responses by cytokines [14, 16, 136-138] or by DNA vaccines [139], suggesting that vaccines specifically formulated to include or induce cytokines are likely to be of great utility in protecting the aged population. It is not known, however, if these strategies can also improve B cell functions, which are also significantly down-regulated during the aging process. More than 10 years ago, a genetically engineered nontoxic vaccine adjuvant, targeting B cells with the immunomodulating cholera toxin A1 subunit, has been described to be effective in directly stimulating naïve B cells to differentiate into GC B cells able to produce antibodies of all subclasses [140]. More recent results from a company working in the field of vaccinology have shown that MF59, a well-established safe and potent vaccine adjuvant that has been licensed for more than 13 years for use in an influenza vaccine focused on elderly subjects, was able to induce not only influenza-specific CD4+ T cells, but also strong and long-lasting memory T and B cells [141]. Another company in the field of vaccinology has licensed the AS04 adjuvant, which elicits higher vaccine-specific antibody responses and induces higher levels of memory B cells [142]. Although these results are encouraging, a large amount of work is still needed to fully characterize the molecular and cellular defects occurring with aging in APCs, T and B cells, in order to provide ways to improve vaccine responses in aged individuals.

Conclusions and future avenues

Identification of autonomous B cell biomarkers of aging which affect function include decreased IgG class switch recombination, AID, E47 and its mRNA stability. These were initially reported for mice but have also been extended to human aged B cells. These molecules offer possible targets for intervention either pharmaceutically or via life style changes to improve the humoral immune response in the elderly.

Acknowledgements

This work is supported by NIH AG-17618 and AG-28586 (BBB).

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